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Ultrasonographic, immunological and bacteriological diagnostic work up for subclinical endometritis in Arabian mares

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In this study, uterine lavage samples were collected from 36 Arabian mares and separated into two groups based on the signs. The first group (10 clinically healthy mares). The 2nd group (26 mares suspected to be affected with endometritis). Bacteriological examination of the samples, revealed that the most frequently isolated bacteria was *Streptococcus equi* spp.(25%). Total protein, TAC, IL-6, Lysozyme, Nitric Oxide (NO) and LDH levels were measured for each animal. The results showed a significant rise in the values of IL-6, Lysozyme, NO and LDH with a slight increase in the total protein values of the samples obtained from the suspected group. The relationship between bacteriological results and results of ultrasonography, revealed that, the levels of isolated bacteria were directly proportional to the degree of edema and the amount of fluids in the uteri of Arabian mares suspected to be affected with endometritis. Furthermore, a very strong significant elevation in the concentrations of IL-6, NO, LDH and Lz was found in samples obtained from mares in the suspected group with a degree of edema (from ++ to +++) and levels of intrauterine fluids (from 3 to 15 mm) when compared to their concentrations in samples of clinically normal Arabian mares. In the current investigation, we used UPGMA clustering dendrogram analysis to compare the protein fingerprints of the uterine lavage samples from clinically normal Arabian mares and Arabian mares suspected to be affected with endometritis, and the analysis revealed a moderate similarity between them with a percentage of (60%).

Keywords: endometritis- immunological- dendrogram- Arabian mares- interleukin

INTRODUCTION

Endometritis is a major cause of female infertility, affecting up to 15% of broodmares. But because it frequently lacks clear clinical signs, it often goes undiagnosed. This invisible and often undiagnosed -disease affects the delicate lining of a mare's uterus—the endometrium—which can become inflamed and create a hostile environment for sperm, as well as any resulting embryo, to live. Thus, often the only clinical sign of endometritis is not what you do see but what you don't see. (Christa Lesté-Lasserre 2010).

The lack of efficient procedures and diagnostic tools to establish a correct diagnosis of

mares with subclinical endometritis impede the initiation of relevant treatment, leading to suboptimal management of the mare (LeBlanc and Causey 2009).

Evidence suggested that fertility of mares was impaired if the endometrial culture was positive and that, the most common etiologic agent of endometritis was *Streptococcus zooepidemicus*, but several other organisms may be involved, including *Escherichia coli*, *Pseudomonas aeruginosa*, and fungi are incriminated in some cases, particularly in mares with reduced resistance. (Robert and Gilbert 2014).

Total antioxidant capacity (TAC) is an analyte

frequently used to assess the antioxidant status of biological samples and can evaluate the antioxidant response against the free radicals produced in a given disease.(Camila et al., 2016). Oxidative stress, resulting from excessive production of free radicals in cases of endometritis, reduces (TAC).(Kaya et al., 2017).

Cytokines play an important role in a wide range of reproductive related processes. The complexity of their network regulation is due to unique properties of cytokines, including pleiotropism, where each cytokine has multiple target cells in an array of different organs (Orsi and Tribe 2008). IL-6 is a pleiotropic cytokine which is produced by different types of immune and non-immune cells. Many immune cell types are reported to produce IL-6 including T cells, B cells, and macrophages. Other cell types known to produce IL-6 include skeletal and smooth muscle cells (Carolina et al., 2010).

Lysozymes are hydrolytic enzymes which cleave the glycosidic linkages of bacterial peptidoglycan. They are typically found in exocrine secretions of animals where they act as a primary defense against bacterial infection. Lysozyme activity appears in the uterine secretions of pigs in response to progesterone treatment (Roberts et al., 1976)

Nitric oxide (NO) is a main mediator of smooth muscle relaxation in different organs including the uterus. (Yallampalli et al., 1993 and Bani eta l., 1999) It is synthesized from L-arginine by different isoforms of nitric oxide synthase (NOS).The inducible NOS (iNOS) is typically expressed at sites of inflammation, and produces large amounts of NO for a prolonged time. (Frean et al., 1997 and Ekerhovd et al., 2000).The different isoforms of NOS have been shown to be expressed in a variety of tissues and cell types in many species including the horse (Hughes and Loy 1969).

LDH is a hydrogen transfer enzyme that is found in the cytoplasm of most of the the body cells. Although LDH is abundant in tissue cells, blood levels of the enzyme are normally low. However, when tissues are damaged by injury or disease, they release more LDH into the blood stream (Judith E.2017).

Tissue-specific protein profile is determined by its function, structure, intensity of metabolism and usefulness. This profile remains under hormonal control. Any disturbance in the general metabolism may be reflected in changes in both protein quantity and quality. These changes can

be of low or high specificity, and some can be used as clinical markers of pathological conditions.(Kankofer et al., 2014)

The aim of this study was to provide an integrated overview about different diagnostic methods for endometritis in Arabian mares and to try to identify some potential markers useful in endometritis diagnosis.

MATERIALS AND METHODS

Experimental design:

Briefly, 36 Arabian mares (from 4 to 19 years age) were separated into two groups based on the signs. The first group consisted of ten mares with no symptoms of endometritis, their results were taken as control values. The second groups (comprised 26 mares) were those that had been bred three or more times unsuccessfully in the same breeding season without clear clinical evidence of inflammation, just increased degree of uterine edema.

Reproduction Examination:

Ultrasonography was performed using real time B-mode scanners (Sonoscape -China) equipped with 4-7.5 MHz frequency linear-array rectal transducer.

Sampling:

Low volume uterine lavage was applied according to (Leblanc et al., 2007).

Bacteriological examination and haemolytic activity:

Vaginal swabs samples were bacteriologically examined according to (Markes et al., 2013) and the suspected colonies were smeared according to (Quinn et al., 1994).

Immunological examination :

A-Estimation of Total protein:

Estimation of total protein: was carried out calorimetrically by the method of (Henry et al., 1974).

B-Determination of horse IL-6 concentration :

It was done according to (Katarzyna et al., 2015).

C-Determination of TAC:

It was measured according to (Trachootham et al., 2008)

D-Detection of lysozyme concentration:

Lysozyme assaying was done according to (Schultz 1987).

E-Measurement of nitric oxide (NO) concentration:

It was assessed according to the assay described by (Rajarman et al., 1998).

F-Estimation of LDH:

It was done according to (Buhl & Jackson, 1978).

G-Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE):

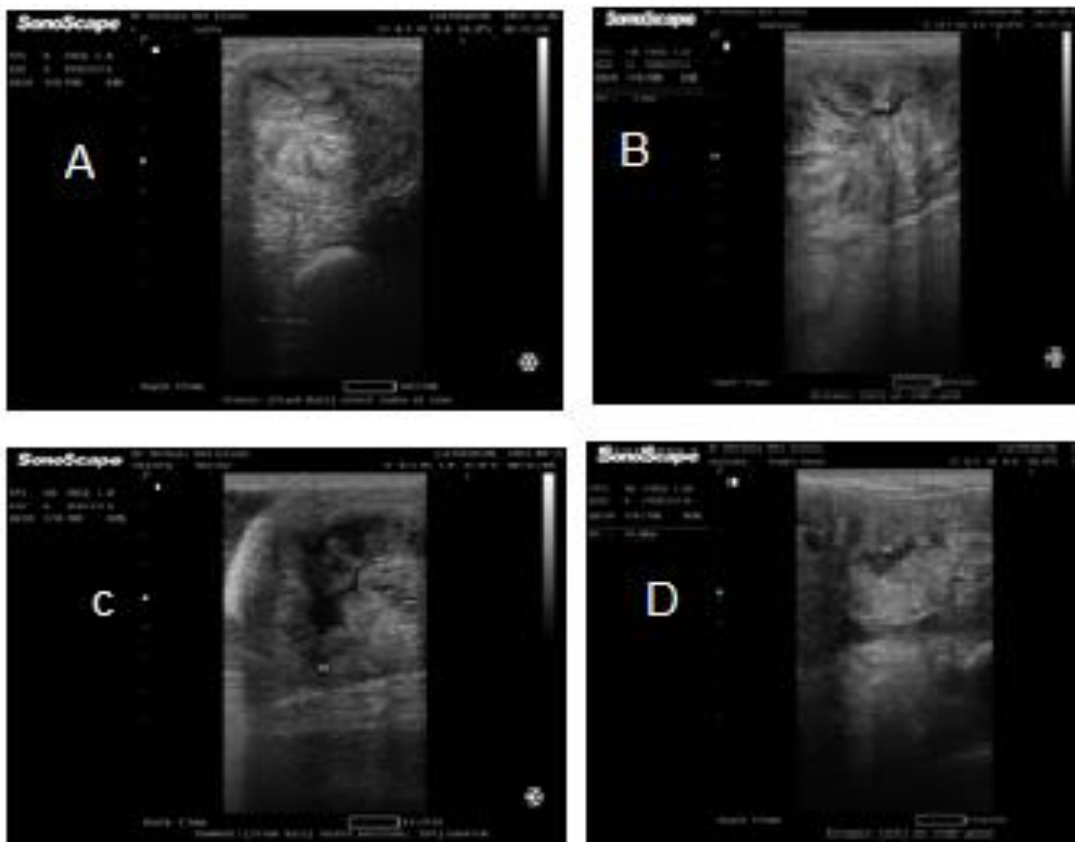
The uterine lavage samples were denatured and separated in 10% sodium dodecyl sulphate-30% polyacrylamide separating gel electrophoresis according to the method of (Laemmli 1970).

Statistical analysis:

Collected data were analyzed for the mean and standard error of mean. Significance of the results was evaluated by (analysis of variance (ANOVA)) according to (Petrie and Watson 1999).

RESULTS

Figure. (1) Ultrasonography scanning of examined mares.



(A) Normal uterine oedema (+) in normal cycling mares of control group.

(B) Grade (++) oedema of the uterine horn with scanty amount of clear fluid (4 mm thickness).

(C) Grade (+++) uterine oedema in the uterine horn and (D) uterine oedema in the uterine body (10 mm thickness).

Table (1) Prevalence of single infections in examined uterine lavage samples from of examined mares

Total number of examined mares	Isolated Bacteria														Bacteriologically negative samples	
	Total positive		<i>Streptococcusequi.</i>		CNS		<i>E.coli</i>		<i>Enterococcus spp.</i>		<i>Pseudomonas aeruginosa</i>		Fungi		No	%
	No	%	No	%	No	%	No	%	No	%	No	%	No	%		
36	26	72.2	9	25	3	8.3	5	13.9	1	2.75	1	2.75	7	19.5	10	27.8

Table (2) Total protein composition of examined uterine lavage samples:

	Control group Clinically normal Arabian mares (n=10)	Arabian mares suspected to be affected with endometritis (n=26)
Total protein (gm/L)	4.42 ± 0.01	4.51 ± 0.08

Table (3): Levels of Total anti-oxidant capacity (TAC) and interleukin 6 (IL-6) in uterine lavage samples of healthy Arabian mares and those suspected to be affected with endometritis:

	Clinically normal Arabian mares (n=10)	Arabian mares suspected to be affected with endometritis (n= 26)
Total antioxidant capacity (TAC)(mM/ml)	0.11 ± 0.03*	0.04 ± 0.01
Interleukin6 (IL-6) (Pg/ml)	30.32 ± 3.22	94.22 ± 6.41*

*Significantly different from control at p < 0.05

Table (4) Lysozyme, NO, and LDH composition in examined uterine lavage samples

	Clinically normal Arabian mares (n=10)	Arabian mares suspected to be affected with endometritis (n= 26)
Lysozyme (µg/ml)	2.3 ± 0.1	3.49 ± 0.25*
NO (µM)	3.1 ± 0.2	5.27 ± 0.66*
LDH IU/L	10.25 ± 1.1	18.9 ± 1.37*

*Significantly different from control at p < 0.05

Table (5): Relationship between bacteriological results and results of ultrasonography

	Oedema	Fluids	Bacteriological results					
			CNS	<i>Streptococcusequi</i>	<i>E.Coli</i>	<i>Enterococcus spp.</i>	<i>Pseudomonas spp</i>	<i>Candida albicans</i>
Clinically normal Arabian mares (n=10)	+	-	Negative for bacteriological growth					
Arabian mares suspected to be affected with endometritis (n=26)	++	3-10 mm	2	4	3	1	-	4
	+++	10-15 mm	1	5	2	-	1	3

Table (6): Relationship between the levels of studied uterine lavage immunological components and results of ultrasonography

	Oedema	Fluids	NO (µM/ml)	LDH (IU/L)	Lz (µg/ml)	Total protein (gm/L)	IL-6 (Pg/ml)	TAC (mM/ml)
Clinically normal Arabian mares (n=10)	+	-	3.1± 0.2	10.25± 1.1	2.3 ± 0.1	4.42± 0.01	30.32 ± 3.22	0.11 ± 0.03*
Arabian mares suspected to be affected with endometritis (n=26)	from++ to+++	from 3 to 15 mm	5.27 ± 0.66*	18.9 ± 1.37*	3.49± 0.25*	4.51 ± 0.08	94.22 ± 6.41*	0.04 ± 0.01

*Significantly different from control at p < 0.05

Figure (2): Electrophoretic protein profiles of different uterine lavage samples from clinically normal Arabian mares and Arabian mares suspected to be affected with endometritis.

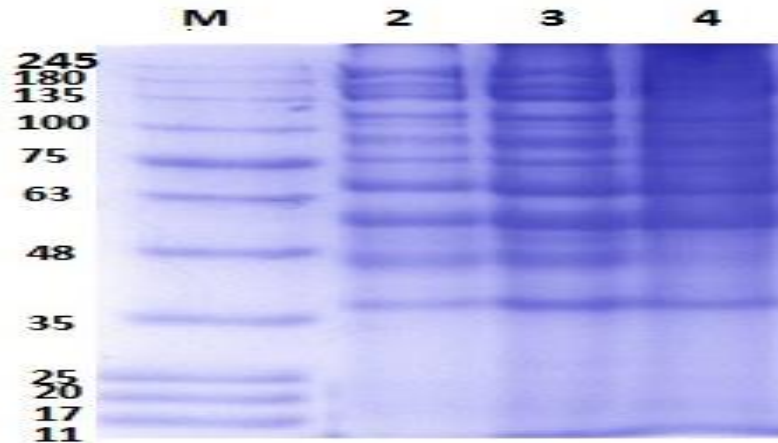
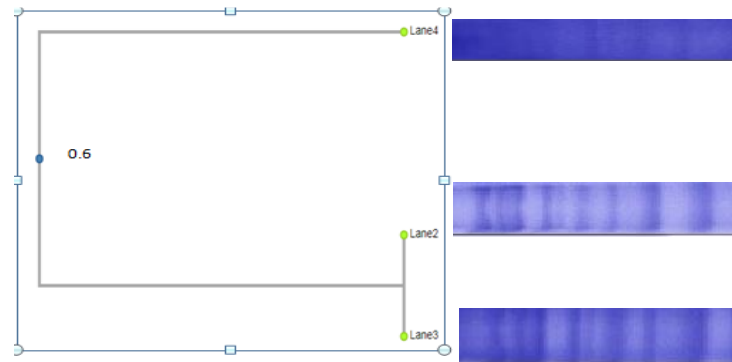


Figure (3): Dendrogram analysis of the uterine lavage protein bands from clinically normal Arabian mares and Arabian mares suspected to be affected with endometritis.

Figure



M: marker

Lane (2&3): uterine lavage samples from clinically normal Arabian mares

Lane (4): uterine lavage samples from Arabian mare suspected to be affected with endometritis.

DISCUSSION

In this study, transrectal ultrasonography (US) has been used to evaluate the grades of oedema as well as the intrauterine fluid accumulation in the animals under study. Normal control mares showed grade (+) oedema with no fluids accumulation or evidence of inflammation (Fig. 1A), while mares suffered from subfertility showed grades (++) , (+++) of oedema with scanty amounts of fluids (Fig.1B, 1C and 1 D) and this group of animals was suspected to be affected with endometritis. Accordingly, further examination of the suspected group of mares should be conducted. It seems that, (Camilla et al., 2015) had a similar observation , the authors suggested that, an altered endometrial oedema pattern in a mare showing increased oedema could therefore imply a subclinical uterine infection, and therefore, further investigations are required to uncover the pathogenesis of subclinical infections

In the ongoing work, bacteriological examination of the uterine lavage samples revealed that the most frequently isolated bacteria was *Streptococcus equi* (25%) followed by *Candida albicans* (19.5%), *E.coli* (13.9%), *CNS spp.* (8.3%), *Enterococcus spp.* and *Pseudomonas aeruginosa* (2.75%). These results agreed with (Skive et al., 2017) who recorded that, *S.zooepidemicus* was the most frequent

cause of infectious endometritis in mares.

In the present study, the total protein composition of uterine lavage samples from Arabian mares suspected to be affected with endometritis showed a slight increase than that of clinically normal Arabian mares. This result came in accordance with the results of (Selvaraj et al.,2003), who considered the elevation of the total protein concentration in the uterine lavage samples of the examined mares as an evidence for uterine inflammation and bacterial infection in 4 cases and evidence for uterine infection without inflammation in 2 cases. This slight increase may be referred to the elevation of the levels of damaged tissues, lysed microorganisms or cellular debris.

One of the goals of this study was to assess the association between endometritis in Arabian mares and TAC levels. To the best of our knowledge, this is the first study that examined the association between the TAC levels in mares uterine lavage samples and the endometritis. The results showed that the TAC levels significantly decreased (at $p < 0.05$) in uterine lavage samples of Arabian mares suspected to be affected with endometritis than clinically normal Arabian mares. This significant decrease may be attributed to the over consumption of the total antioxidants during the protection against the invading organisms to detoxify the released reactive oxygen species (ROS). Similarly (Behera et al., 2016) reported

significantly lower TAC levels in the follicular fluid of buffalo with purulent endometritis when compared to that of non-endometritis. At the same time, results of (Baithalu et al., 2017) observed low concentrations of TAC during peripartum period in cows that developed postpartum uterine infection than normal cows.

Data presented in table (3) revealed a significantly higher levels of IL-6 (at $P < 0.05$) in uterine lavage samples of mares with suspected endometritis when compared to clinically healthy mares. (Marta et al., 2016) reported that, concentrations of IL-6 were increased in mares exhibiting sub-acute suppurative endometritis (SSE), when compared with unaffected mares. Likewise, (Li.De-jun et al., 2010) concluded that, IL-6, CRP, Hp and SAA could be the sensitive index for diagnosis of endometritis in cows.

Since endometritis is an invisible disease (where clinical signs of inflammation are absent), tests for measuring some inflammatory components and antioxidants (Lysozyme, LDH and NO) in uterine lavage samples of suspected mares were conducted in the present investigation as a trial for recognition or diagnosis of endometritis in mares. (Table 4).

(Stephanie et al., 2017) mentioned that, lysozyme was the cornerstone of innate immunity. The mechanism for bacterial killing by lysozyme occurs through the hydrolysis of cell wall peptidoglycan (PG). In addition to its direct antimicrobial role, more recent evidence has shown that lysozyme modulates the host immune response to infection. The on-going research (Table 4) showed a significant increase (at $p < 0.05$) in the levels of Lysozyme in the uterine lavage samples of the Arabian mares showing clinical signs of endometritis when compared to clinically normal Arabian mares. Our results came along with (Reilas&Katila2002) who found that high concentration/ activities of lysozyme in uterine fluid of mares was associated with uterine inflammation.

In the current study, (Table 4) LDH recorded a significant elevation (at $p < 0.05$) in uterine lavage samples of Arabian mares suspected to be affected with endometritis when compared to that of clinically normal Arabian mares. These findings agreed with (Pycock and Allen 1990), who found that lactate dehydrogenase and Lysozyme were present in flushings from non-infected uteri, but their concentrations increased with time after intrauterine infusions of *Streptococcus zooepidemicus* in genitally normal mares during oestrus.

In this work, The significant increase in NO concentration (at $p < 0.05$) obtained from uterine lavage samples of Arabian mares suspected to be affected with endometritis was obvious when compared to samples of clinically normal Arabian mares (Table 4). Similar results were obtained by (Woodward et al., 2013 and Khan et al., 2018), who demonstrated a significantly greater endometrial NO levels in mares susceptible to persistent breeding-induced endometritis (PBIE) than resistant mares, which appeared to be as a result of increased iNOS expression. Furthermore, results of (SongXue et al., 2015) showed that the concentration of NO was significantly higher ($P < 0.01$) in uterine secretions with subclinical endometritis (SCE) when compared to those of normal cows.

Regarding the relationship between the bacteriological results, levels of studied uterine lavage Immunological components and the results of ultrasonography, table (5 and 6) showed that: 1) The most common isolated bacteria were *Streptococcus equi*. 2) The numbers of the isolated bacteria were directly proportional to the degree of oedema and the amount of fluids in the uteri of Arabian mares suspected to be affected with endometritis. 3) A very strong significant elevation (at $P < 0.05$) in the concentrations of NO, LDH, Lz and IL-6 in samples obtained from mares suspected to be affected with endometritis, with a degree of oedema (from ++ to +++) and levels of intrauterine fluids (from 3 to 15 mm) when compared to their concentrations in samples from clinically normal Arabian mares. 4) A significant decrease (at $P < 0.05$) in the concentrations of TAC with the same degree of oedema and the same levels of intrauterine fluids. These results may lead us to a conclusion that, bacteriological culturing and the quantification of IL-6, TAC, NO, Lz, Total protein and LDH, in the uterine lavage samples of mares suspected to be affected with endometritis could be valuable diagnostic and prognostic aids. These findings may come along with the findings of (Mohamed et al., 2015), who said that, When ultrasonography is combined with other investigative methods, e.g. endometrial cytology, isolation of the causative bacteria and antibiotic sensitivity tests will eminently increase its accuracy of detection and consequently the manipulation decisions in Arabian mares.

One of the purposes of the current investigation was to compare between the protein profile of uterine lavage samples obtained from mares suspected to be affected with endometritis

and that obtained from clinically healthy mares in a trial to understand and to determine possible susceptibility protein markers that could indicate subclinical endometritis in mares. For this purpose, we used UPGMA clustering dendrogram analysis to compare the protein fingerprints of the uterine lavage samples from clinically normal Arabian mares and Arabian mares suspected to be affected with endometritis. (Fig.3). Unfortunately, the protein dendrogram illustrated that there were moderate similarities (60%) between the protein fingerprints of the uterine lavage samples from clinically normal Arabian mares and those from Arabian mares suspected to be affected with endometritis. This may be attributed to the change in the protein structure of the samples obtained from mares suspected to be affected with endometritis due to the presence of some inflammatory and pro inflammatory proteins such as cytokines. For this reason we couldn't depend on this technique to determine possible susceptibility protein markers that could indicate subclinical endometritis in mares.

CONCLUSION

We concluded that, Arabian mares that fail to conceive, without showing typical signs of clinical endometritis, should be suspected of subclinical endometritis (SE). Furthermore, Ultrasonography in broodmares was a reliable field diagnostic technique that enables the equine clinician to perform non-invasive examination of the uterus, but it's better to be combined with other investigative methods. In this work, levels of IL-6, LZ, NO and LDH were significantly increased (at $P < 0.05$), beside this, a significant decrease in the level of TAC (at $P < 0.05$) was observed in mares suspected to be affected with endometritis. This study offers an opportunity for developing the diagnosis strategies in Arabian mares that were apparently healthy when diagnosed by ultrasonography in a way that eminently increases its accuracy.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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